2014

Characterization of Putative Defense Genes in Nonvascular Plants

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4/28/14 Date

Certified by Judith Harper Marrick — 5/13/14 Date
Director, Honors Program

For Honors Program use:

Level of Honors conferred: University Magna Cum Laude

Departmental High Honors in Biology

Characterization of Putative Defense Genes in Nonvascular Plants

A Thesis

Presented to the Department of Biology

College of Liberal Arts and Sciences

and

The Honors Program

of

Butler University

In Partial Fulfillment

of the Requirements for Graduation Honors

Blaine Harlan

4-28-2014

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Abstract:

Vascular plants have many known defenses against herbivory and pathogen infection. One inducible defense system that has been extensively studied in vascular plants is systemic acquired resistance (SAR), which is a plant-wide response that results in resistance to a wide range of pathogens Many genes that play a role in SAR have been characterized. Although several studies of plant-pathogen interactions in non-vascular plants have occurred, it was not until recently that the existence of SAR was shown in these plants. The goal of the present study was to confirm the presence of homologous defense genes in moss, and to study their expression following pathogen inoculation.

Chapter 1: Introduction

Defense in vascular plants

There are many ways in which plants are able to protect themselves from pathogen attack or herbivory. The first forms of defense vascular plants employ are physical structures such as waxy cuticles, trichomes, or thorns (Tang 2007). In addition to these constitutive defenses, a couple inducible defense systems also exist. In vascular plants these are known as the hypersensitive response (HR) and a system acquired resistance (SAR) mechanism against pathogens (Edreva 2005).

Hypersensitive Response in Vascular Plants

The hypersensitive response is characterized by a type of programmed cell death (PCD), most likely to restrict pathogen growth (Greenberg and Yao 2004). Plants use reactive oxygen species (ROS) produced during metabolism in the mitochondria and other plastids to initiate HR (Lam et al. 2001). Once the plant is wounded, it is susceptible to many pathogens. However, plants have the ability to sense a pathogen before wounding as well. These pathogens include viruses, that hijack the host's cells to produce more viruses, and bacteria and fungi, which can use the nutrients of the cells to grow and reproduce. PCD is an effective response to damage of the cell wall since it stops the pathogen's ability to infect the tissue and proliferate while only sacrificing a few cells.

Systemic Acquired Resistance in Vascular Plants

Plants do not have an immune system like animals. Instead of specific recognition of infection like the T-cells in animals, plants use a broad spectrum approach to recognizing and reacting to pathogens. SAR is characterized by an enhanced ability to react to a pathogen attack following initial exposure to a pathogen. This enhanced ability is called "priming" and it occurs not only in the region that was initially attacked but in tissues in other regions of the plant as well (Durrant and Dong 2004). The SAR is a broad resistance response to infection of many pathogens including viruses, bacteria, oomycetes, and fungi. Once the plant is initially attacked, there is a signal sent from the site of infection to other tissues. The exact identity of the signal is unknown, but it is hypothesized to be a lipid signal (Bate et al. 1998). Other studies have supported the idea that ethylene, a simple gas, or hydrogen peroxide, a reactive oxygen species, as possible components of SAR signaling (Bate et al. 1998). Once SAR is activated, salicylic acid (SA) is needed to establish SAR in distal tissues and activate defense genes there (Ryals et al. 1996). The cells are then primed to prevent another attack in the future, the exact mechanism of how cells do this is not entirely understood. It is believed that priming may involve accumulation of cellular components important in signal transduction and amplification (Conrath 2006). Another study suggests that priming may also include histone acetylation of defense genes (Ng et al. 2005). These activities would not activate the defense genes, but would make the process of responding to an

attack much quicker. Constitutive expression of defense genes is not an effective response due to the high energy cost of making the defense proteins (Conrath 2006).

Pathogenesis-related genes

Plants sense infection by signals from the cell wall of the pathogen or the pathogen itself. (Ponce de Leon et al. 2007) Pathogenesis-related (PR) genes are expressed when the plant senses an infection (Nawrath and Metraux 1999). There are many different ways in which PR genes are induced, some of which are wounding, SA, fatty acids, in organic salts, low temperature, osmotic shock, ethylene, jasmonates, abscisic acid, auxin, etc. (Edreva 2005). PR genes encode for proteins that have anti-fungal or anti-bacterial properties act in cell wall reinforcement, or have other defensive properties. There are seventeen families of PR genes identified in vascular plants so far (table 1). The function of PR-1 has not been fully described, but it is often used as a marker to confirm the presence of SAR (Loon et al. 2006). It has been proposed that PR-1 may encode for proteins related to redox reactions with reactive oxygen intermediates. These intermediates may act as local secondary messengers after SA (Maleck 2000). PR-2 genes encode for β-1,3-glucanase which is an enzyme that catalyze endo-type hydrolytic cleavage of glucosidic linkages in β-1,3-glucan (Loon et al. 2006). β-1,3-glucans are found in the cell wall of some fungi, oomycetes, and bacteria. PR-3 and PR-4 genes encode for chitinases. Chitinases are enzymes that cleave poly-β-1,4-Nacetylglucosamine (chitin) (Loon et al. 2006). PR-3 and PR-4 genes would target fungi,

some oomycetes, and possibly herbivorous insects and nematodes since these organisms use chitin as a structural component (Loon et al. 2006). PR-5 genes are characterized as thaumatin-like because the proteins produced closely resemble the protein thaumatin. These genes encode for proteins that have been shown to be related to defensive activity against mainly oomycetes but other pathogens as well (Loon et al. 2006). A basic PR-5 protein is osmotin, which is induced by osmotic stress, can perform anti-oomycete activity. Osmotin has also been seen inducing apoptosis in yeast by binding to phosphomannans in the cell wall.

Other Defense Genes

In addition to the PR genes, other genes are also induced upon attack. Some of the main defense related genes are chalcone synthase (CHS), phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX). CHS is a defense related gene that is the first enzyme in the pathway to producing flavonoids, which are known to have anti-microbial and anti-fungal properties (Ponce de Léon et al. 2007). PAL mediates the biosynthesis of phenylpropanoids and salicylic acid (SA). As mentioned earlier, SA has been shown to play a part in signaling in SAR. LOX is a key enzyme in the synthesis of defense related compounds including jasmonic acid (JA) and SA. LOX is also thought to be responsible for activation of PAL (Ponce de Léon et al. 2007).

Table 1. Known PR gene families (Loon et al. 2006).

Family	Type member	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	B-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco "R"	Chitinase type I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tobacco Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P_{6g}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR-10	Parsley "PR1"	"ribonuclease-like"
PR-11	Tobacco class V chitinase	Chitinase type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	*Arabidopsis* THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tabacco PRp27	Unknown

Plant evolution

Photosynthetic green algae are the common ancestor of land plants. There are a wide variety of land plants, these plants can be split into vascular plants and non-vascular plants (Fig. 1). The move to land included many risks to plants including heat, dessication and damage by UV rays. Despite the harsh environment, land contained rewards that aquatic life did not such as plentiful CO_2, sunlight and few competitors. Because of the move from water to land, many adaptations seen in land plants are geared toward surviving the harsh environment of land. Three phyla represent nonvascular plants Bryophyta (mosses), Hepatophyta (liverworts) and Anthocerotophyta (hornworts). The nonvascular plants are not fully independent from water and still require water for reproduction. Mosses are different from the other nonvascular plants in that they possess a rudimentary water conducting tissue. Vascular plants are much more efficient in conducting molecules around the plant. They use xylem elements to transport water and phloem cells to transport carbohydrates. The first four phyla of the vascular plants are the seedless vascular plants Pteridophyta (ferns), Psilophyta (whisk ferns), Sphenophyta (horsetails) and Lycopodiophyta (club mosses). These four phyla still depend on water for fertilization, but have fronds and sori containing sporangia. The last five phyla are characterized by having seeds. These phyla are Coniferophyta, Cycadophyta, Gnetophyta, Ginkgophyta and Angiosperms. Angiosperms in particular are characterized by possessing flowers (McGraw Hill).

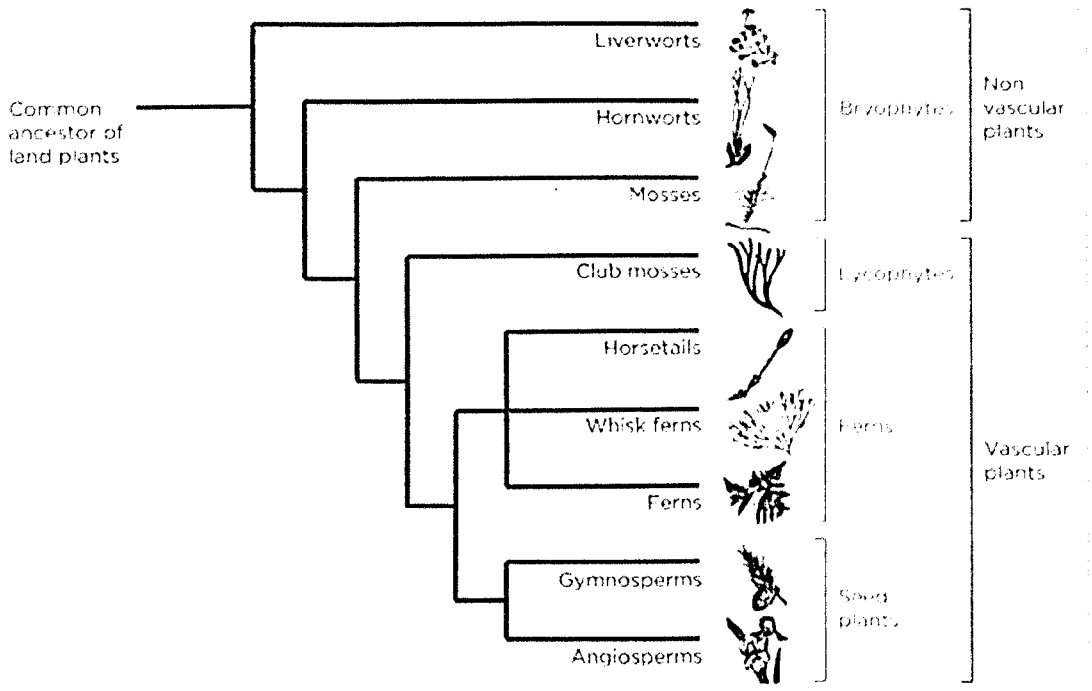

Figure 1. Phylogenetic tree of land plants. Nonvascular plants, such as mosses, do not have a developed vascular system. Over time new adaptations, like vascular systems, seeds and flowers gave plants the ability to colonize different parts of the land (www.sciencelearn.org)

Structure/morphology of moss

Mosses possess a gametophyte-dominated life cycle, which means that the gametophyte generation is the photosynthetic part of the life cycle (Fig. 2). The male and female gametophytes will produce flagellated sperm and eggs respectively. These primitive land plants still require water to reproduce. The egg is retained in the female gametophyte and becomes the sporophyte once fertilized. The sporophyte is dependent and attached to the gametophyte. The sporophyte produces spores which then land and create protonema. The gametophytes then grow from the protonema. The gametophyte of mosses are generally small that occur in colonies that form mats. Mosses are attached to the substrate with rhizoids, filamentous branched root-like structures. Rhizoids are not sites for uptake of water or nutrients, but function mainly as attachment. *Physcomitrella patens*, the model system used for studies of nonvascular plants, adopts a clumped growth pattern (Fig. 3). *Amblystegium serpens*, another moss species used in this study, grows in a prostrate creeping form (Fig. 3). The most conspicuous way in which moss differs from vascular plants is that they do not have a vascular system. This is a very important difference since the vascular system is a main way chemical signals are passed through the plant. Vascular plants are able to send messages relatively quickly across tissues and organs. One of the main questions is, if mosses do have SAR, how are they able to send the signals to other tissues without a true vascular system? (Structure and Anatomy from review by Crandall-Stotler and Bartholomew-Began 2007)

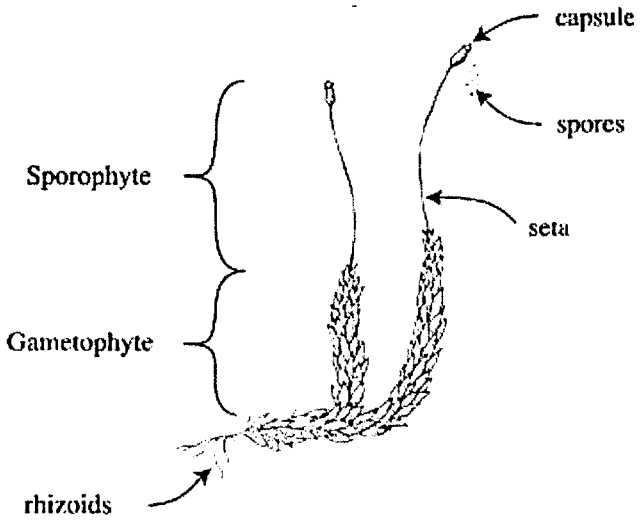

Figure 2. Generalized model of moss morphology. The gametophyte is the main vegetative structure in the moss life history. The sporophyte generation is dependent on the photosynthesis of the gametophyte to survive and gives rise to the spores. The spores then grow into the protonema and continue the life cycle.

(www.kentuckyawake.org)

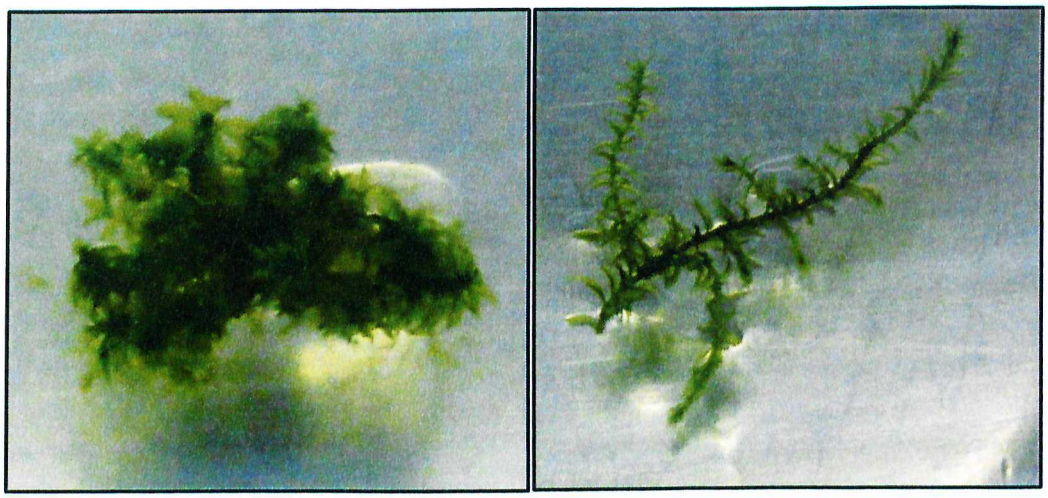

Figure 3. Differing morphologies of the gametophyte stage of *Physcomitrella patens* (left) and *Amblystegium serpens* (right). *P. patens* has a bushy morphology, while *A. serpens* has a creeping prostrate morphology.

Plant/pathogen interactions in moss

The defense mechanisms of vascular plants have been widely studied, however very little research on plant-pathogen interactions has been conducted in nonvascular plants. It is known that mosses have an HR and most likely have SAR. The hallmark of HR is cytoplasmic shrinkage of the infected area. Shrinkage was observed in *P. patens* when infected by either of the two necrotrophic pathogens, *Erwinia carotovora* and *Botrytis cinerea* (Ponce de Leon et al. 2007). One of the signaling molecules used by SAR is SA. Andersson et al. (2005) Performed a study on *Physcomtrella patens* and show that there is a SA-dependent defense system. In addition to the defense system induced by SA treatment, PR-1 was also identified in *P. patens*. Three defense genes, CHS, LOX and PAL, were also identified in *P. patens* (Oliver et al. 2009). More recently, Butler graduate Peter Winter confirmed the existence of SAR in moss by showing that primary inoculation of a moss with a pathogen results in increased systemic resistance to future infections (Winter et al., submitted).

Defense genes in moss

Only one homolog of the PR families, PR-1, has been shown to be expressed in the moss *Physcomitrella* (Ponce de leon et al. 2007). The function of PR-1 has not been fully described, but it is often used as a marker to confirm the presence of SAR (Loon et al. 2006). Three defense genes, CHS, LOX and PAL, were also identified in *P. patens* (Oliver et al. 2007). As mentioned earlier, CHS is a defense related gene that is involved in

the production of flavonoids, which are known to have anti-microbial and anti-fungal properties. PAL mediates the biosynthesis of phenylpropanoids and salicylic acid (SA). As mentioned earlier, SA has been shown to play a part in signaling in SAR. LOX is a key enzyme in the synthesis of defense related compounds including jasmonic acid (JA) and SA. LOX is also thought to be responsible for activation of PAL.

Thesis Statement

It is possible that SAR evolved before the divergence of vascular and non-vascular plants, so the mechanism should be conserved in all plants. In order to support this hypothesis more work needs to be done to compare SAR related genes in vascular plants to those in non-vascular plants. The existence of homologous defense genes in non-vascular and vascular plants would support the hypothesis that SAR evolved before their divergence. If non-vascular plants show a different set of genes for SAR, then it is possible that SAR evolved in both types of plants after divergence due to convergent evolution. This study focused on identifying defense-related genes in *A. serpens* and *P. patens* and also determining expression of putative defense-related genes following treatment of the moss with an elicitor. Some defense genes that I anticipate to identify in mosses are PR-1 through PR-6 as well as CHS and PAL. I hypothesize that the genes will be upregulated throughout the plant quickly after pathogen challenge.

Literature Cited

Andersson, R. Akita, M. Pirhonen, M. Gammelgard, E. Valkonen, J. 2005. Moss-*Erwinia* pathosystem reveals possible similarities in pathogenesis and pathogen defense in vascular and nonvascular plants. *J. Gen. Plant Path.*71: 23-28.

Bate, N. Rothstien, S. 1998. C_6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. The Plant Journal 16:561-569.

Conrath, U. 2006. Systemic acquired resistance. *Plant Signaling & Behavior* 4: 179-184. Review.

Crandall-Stotler, B. J., S. E. Bartholomew-Began. 2007. Morphology of mosses (Phylum Bryophyta). *Flora North America* 27: 3-13. Review.

Durrant, W. and Dong, X. 2004. Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42:185-209. Review.

Edreva, Aglika. "Pathogenesis-Related Proteins: Research progress in the last 15 years." *General Applied Plant Physiology* 31.1-2, 27 May (2005): 105-24. Review.

Greenberg, JT. Yao, N. 2004. The role and regulation of programmed cell death in plant-pathogen interactions. *Cell Microbiol.* 6: 201-211.

Lam, E. Kato, N. Lawton, M. 2001. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* 411: 848-853. Review.

Loon, L.C. van, M. Rep, C.M.J Pieterse. "Significance of Inducible Defense-Related Proteins in Infected Plants." *Annu. Rev. Phytopathol*, March (2006): 44: 135-62. Review.

Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, et al. 2000. The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* 26:403–10

McGraw Hill. Chapter 37: Evolutionary History of Plants. [PDF File] http://highered.mcgraw-hill.com

Nawrath, Christiane and Jean-Pierre Métraux. "Salicylic Acid Induction–Deficient

Mutants of *Arabidopsis* Express PR-2 and PR-5 and Accumulate High Levels of Camalexin after Pathogen Inoculation" *The Plant Cell*, August (1999).

Ng DWK, Chandrasekharan MB, Hall TC. 2005. Ordered histone modifications are associated with transcriptional poising and activation of the phaseolin promoter. *Plant Cell* 18:119-32.

Oliver, Juan Pablo, Alexandra Castro, Carina Gaggero, Tomas Cascón, and Eric A. Schmelz. "Pythium infection activates conserved plant defense responses in mosses." *Planta* 230, 24 June (2009): 569-79.

Ponce de León, Inés, Juan Pablo Oliver, Alexandra Castro, Carina Gaggero, and Marcel Bentancor. "Erwinia carotovora elicitors and Botrytis cinerea activate defense responses in *Physcomitrella patens*." *BMC Plant Biology* 7.52, 8 Oct. (2007).

Ryals, J. Neuenschwander, U. Willits, M. Molina, A. Steiner, H. Hunt, M. 1996. Systemic acquired resistance. *The Plant Cell* 8:1809-1819. Review.

Tang, C. 2007. The wound response in *Arabidopsis thaliana* and *Physcomitrella patens*. (doctoral dissertation)

University of Waikato. 2007. What is a Plant? www.sciencelearn. org

University of Kentucky.2011. Ferns, Mosses, Horsetails. www.kentuckyawake.org.

Winter, Peter S., Collin E. Bowman, Philip J. Villani, Thomas E. Dolan, and Nathanael R. Hauck. (2011) "Systemic acquired resistance in moss: Further evidence for conserved defense mechanisms in plants" Unpublished Manuscript.

Chapter 2: Defense Gene Identification in Moss

Background/Introduction

Primers are short strands of single stranded DNA that have sequence similarity to a target sequence in the genome. The primers will bind to a place in the genome where the sequences match and this will serve as a starting point for DNA synthesis. This process can be used to amplify certain target sequences using polymerase chain reaction (PCR) with primers specifically designed for that sequence. The PCR product can be then ran out on a gel to determine presence of target sequence and size of product by comparison to a DNA ladder. The target sequence is usually a gene, so designing primers that are sequence-specific can help to determine the presence of a gene in a genome.

Only one homolog of the PR families, PR-1, has been shown to be expressed in the moss *Physcomitrella* (Ponce de Léon et al. 2007). The function of PR-1 has not been fully described, but it is often used as a marker to confirm the presence of SAR. (Loon et al. 2006) Three additional defense-related genes, CHS, LOX and PAL, were also identified in *P. patens* (Oliver et al. 2009). As mentioned earlier, CHS is a defense related gene that is involved in the production of flavonoids, which are known to have anti-microbial and anti-fungal properties. PAL mediates the biosynthesis of phenylpropanoids and salicylic acid (SA). As mentioned earlier, SA has been shown to play a part in signaling in SAR. LOX is a key enzyme in the synthesis of defense related compounds including jasmonic acid (JA) and SA. LOX is also thought to be responsible for activation of PAL. Other PR

genes that are likely to also occur in nonvascular plants are PR-2, PR-3, PR-4 and PR-5. PR-2 genes encode for β-1,3-glucanase which is an enzyme that catalyze endo-type hydrolytic cleavage of glucosidic linkages in β-1,3-glucan (Loon et al. 2006). β-1,3-glucans are found in the cell wall of some fungi, oomycetes, and bacteria. PR-3 and PR-4 genes encode for chitinases. Chitinases are enzymes that cleave poly-β-1,4-Nacetylglucosamine (chitin) (Loon et al. 2006). PR-3 and PR-4 genes would target fungi, oomycetes, and possibly herbivorous insects and nematodes since these organisms use chitin as a structural component (Loon et al. 2006). PR-5 genes are characterized as thaumatin-like because the proteins produced closely resemble the protein thaumatin. These genes encode for proteins that have been shown to be related to defensive activity against mainly oomycetes but other pathogens as well (Loon et al. 2006). A basic PR-5 protein is osmotin, which is induced by osmotic stress, can perform anti-oomycete activity. Osmotin has also been seen inducing apoptosis in yeast by binding to phosphomannans in the cell wall. Other PR genes or defense-related genes may also be present in nonvascular plants.

Since *A. serpens* has not been sequenced, specific primers cannot be made initially. One way to amplify sequences in a species where the genome has not been sequenced is to design primers for a closely related species. This method will only work for genes that are highly conserved. Another way is to design degenerate primers. Designing degenerate primers begins with choosing species that have their genomes sequenced and are closely related to the species the primers will be used in. The amino acid sequences of the target gene are then aligned using a computer program. At least

two highly conserved regions of the aligned sequences are needed to make forward and reverse primers. Within highly conserved regions, there may be bases in which there is variability. Degenerate primers include different bases that can accommodate the variability. For example, a Y in the primer sequence would take the place of either a C or a T.

In this part of my thesis, I attempted to design primers that could be used for analysis of expression of defense genes in *A. serpens*. With the design of primers, the expression of defense genes throughout the *A. serpens* plant following pathogen inoculation could be analyzed.

Methods

PW Primers

The PW primers were designed by recent Butler graduate, Peter Winter. These primers were designed from the available *P. patens* sequences available on ncbi.gov. The program BLAST was used to compare the *P. patens* genome with known defense and PR genes in vascular plants he was able to pull out homologous sequences likely to be the gene of interest. The primers that amplified DNA were considered potential candidates for defense genes in *A. serpens*. The PCR products were isolated and send for sequencing. The PCR products of the candidate primers were then run out on a gel for a longer period of time to separate bands more distinctly. Some PRC products contained multiple bands so four were chosen for PR1, two bands were chosen for PAL, one band each was chosen for PR2 and PR4. Each band was then run out on a gel again to

determine if there was DNA present (fig. 4). Two larger bands for PAL were sent out for sequencing.

Degenerate Primers

Since the primers designed for *P. patens* did not successfully amplify the PR genes in *A. serpens*, degenerate primers were designed. The program BLAST from ncbi.gov was used to find the defense genes from *Selaginella moellendorffii* and *Physcomitrella patens*. Clustalw was used to align sequences to find conserved regions. Degenerate primers were designed from two highly conserved regions of each gene, one region for the forward primer and one for the reverse primer. Primers were designed for PR-1, PR-2, PR-3, PR-5, PR-6, PR-7, PR- 9, PR-11, PR-13, PR-14, PR-15, PR-16, LOX and PAL. The specificity of the primers was confirmed using PCR of *Amblystegium* DNA, followed by sequencing of amplification products. The general procedure for PCR was followed.

Sequences & specific primers

PCR products showing a consistent clear band were cut out and prepared for sequencing. CHS and two bands from PAL were sequenced. The specific primers for *A. serpens* were designed from regions of high confidence from the sequence information.

Only one PAL sequences had enough regions of high confidence to design accurate primers.

Redesigned Physcomitrella primers

Primers for *P. patens* were redesigned using the NCBI website to find the target genes in *Arabidopsis*. The BLAST program was used to pull *P. patens* sequences out of the database that show sequence similarity to the target sequence. Once these genes were identified, the 'pick primers' option on the NCBI website was used. This program also checks for specificity to the target sequence. Primers were designed for PR-1, PR-2, PR-3, PR-5, PR-6 and CHS.

Results

PW primers

If defense genes are highly conserved, it would be expected that primers made from *P. patens* sequences may have success in amplifying sequences from *A. serpens*. Out of these primers, five showed consistent and clear bands and were considered potential candidates for defense genes in *A. serpens*. The candidates were PR1, PAL, PR2, PR4, and CHS. The next step is to isolate the PCR products and sequence. CHS showed only one band in the PCR product, while the others showed multiple bands indicating that the amplification was not specific. The PCR products of these primers

were then run out on a gel for a longer period of time to separate bands more distinctly. Four bands were chosen for PR1, two bands were chosen for PAL, one band was chosen for PR2 and PR4. Each band was then run out on a gel again to determine if there was DNA present (Fig. 4). Two bands for PAL were sent out for sequencing (Fig. 4).

Degenerate primers

All of the primers were first screened using PCR. If the primers did not show bands, it was assumed that they were not successful in amplifying the DNA. The primers that did show bands, PR-7, PR-14, PR-15 and PR-16, were ran out on a gel again for a longer period of time to allow farther separation of bands in the gel (Fig. 5). Because there are multiple bands for each primer, all of the primers show non-specific binding to the DNA sequence.

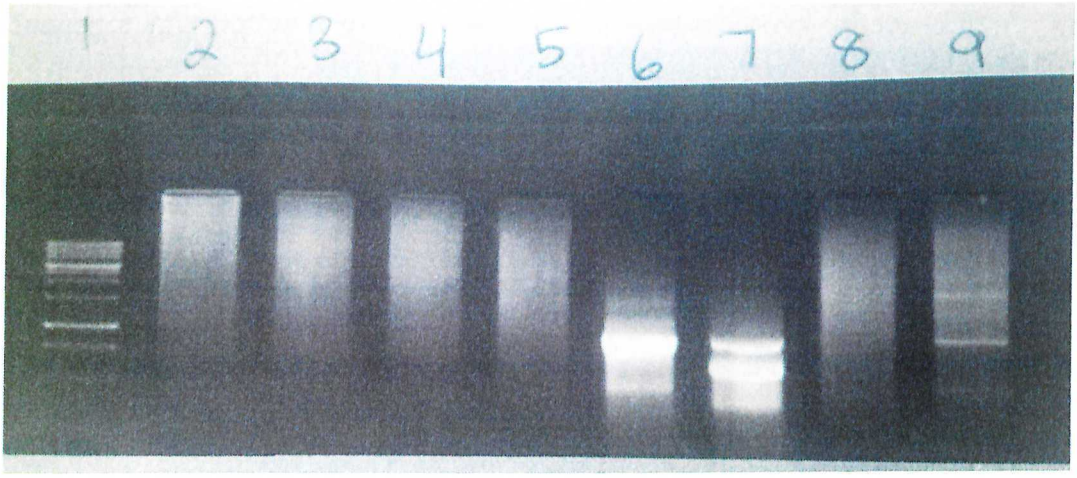

Figure 4. Bands cut out from PCR products of primers amplified in *A. serpens* and run again on gel to determine if DNA was present. Bands 6 and 7 were sent for sequencing. Lane 1 is a 1kb ladder, lanes 2-5 are PR1, lanes 6-7 are PAL, lane 8 is PR2, lane 9 is PR4.

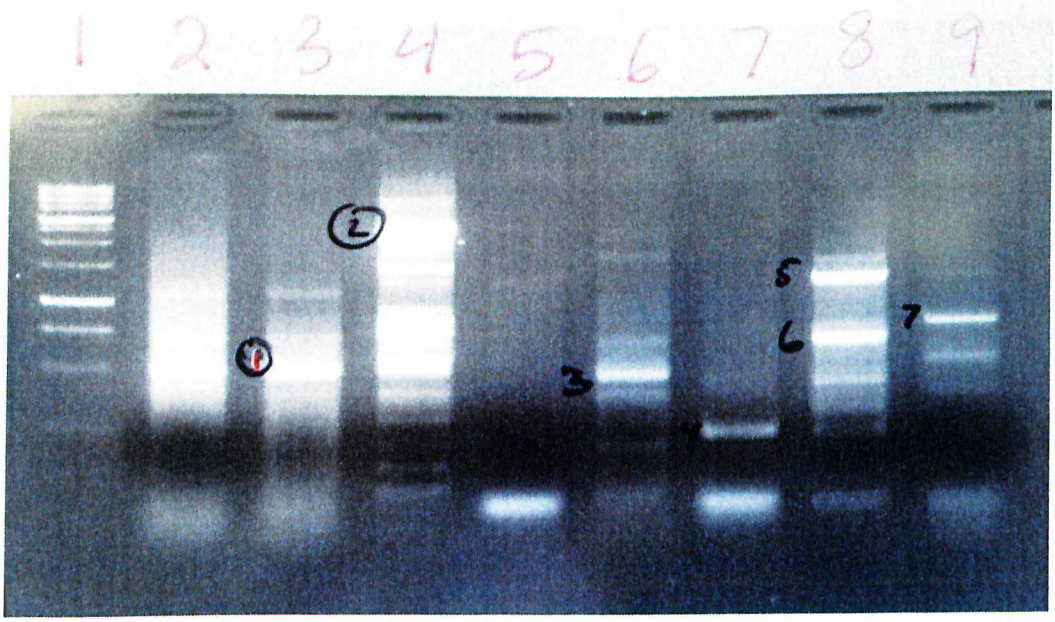

Figure 5. PCR of primers that amplified DNA in a previous screening. Lane 1 is 1kb ladder, lane 2 is PR-7, lane 3 is PR-7, lane 4 is PR-7, lane 5 is PR-7, lane 6 is PR-14, lane 7 is PR-15, lane 8 is PR-16, and lane 9 is PR-16. In some cases multiple primer sets were designed for each target gene.

Sequence information & specific primers

The PCR products using CHS and two PAL primers were sequenced. Figure 6 and 7 below show sequences from *A. serpens* compared to known genes in *P. patens*. The CHS sequence from *A. serpens* was 97% identical to the known CHS gene in *P. patens*. The PAL sequence from *A. serpens* was 90% identical to the predicted PAL gene in *P. patens*.

```
Amblystegium_CHS      VQVPKLAAEAAVKAIKEWGGRKSDITHIVFATTSGVNMPGADHALAKLLGLKPSVKRVMM 60
Physcomitrella        VQVPKLAAEAAQKAIKEWGGRKSDITHIVFATTSGVNMPGADHALAKLLGLKPTVKRVMM 60
                      ********** **********************************************:****** 

Amblystegium_CHS      YQIGCFGGASVLRVAKDLAENNKGARVLAVCSEVTAVTYRAPSENHLDGLVGSALFGDGA 120
Physcomitrella        YQIGCFGGASVLRVAKDLAENNKGARVLAVCSEVTAVTYRAPSENHLDGLVGSALFGDGA 120
                      ************************************************************ 

Amblystegium_CHS      GVYVGXSDP 129
Physcomitrella        GVYVVGSDP 129
                      ****  *** 
```

Figure 6. Amino acid alignment of the CHS protein in *A. serpens* and *P. patens* .

ClustalW was used to align the newly sequenced *A. serpens* CHS gene and the *P. patens* sequence that was found on NCBI. 97% identity of known *P. patens* CHS gene and proposed CHS gene from sequencing in *A. serpens*.

```
Physcomitrella        PFELQPKEGLAMVNGTAVGSALASTTCFDANILAVMAEVLSALFCEVMQGKPEFADPLTH 60
Amblystegium_Pal      PFELQPKEGLALVNGTAVGSALASTVCFDANVLALLAEVLSALFCEVMQGKPEFADPLTH 60
                      ********** ************* ***** **  ************************ 

Physcomitrella        KLKHHPGQMEAAAIMEYLLDGSSYMKAAAKLHETDPLKKPKQDRYALRTSPQNLGPQVEA 120
Amblystegium_Pal      KLKHHPGQMEAAAVMEWVLAGSDFMKAAEKLNETDPLKKPKQDRYALRTSPQNLGPQIEV 120
                      ************* **  * **  **** ** ************************** * 

Physcomitrella        IRNATHSIQREINSVNDNPLIDAAGDRALHGGNFQGTPIGVSMDNMRLAVAAIGKLMFAQ 180
Amblystegium_Pal      IRTATHAIEREINSVNDNPIIDAARGIALHGGNFQGTPIGVSMDNMRLAVAAIGKLMFAQ 180
                      ** *** * ********** ****   ********************************** 

Physcomitrella        FSELVNDFYNNGLPSNLSGGPNPSLDYGMKGAEIAMASYLSELNYLAN 228
Amblystegium_Pal      FSELVNDYYNNGLPSNLSGGPNPSLDYGMKGAEIAMASYLSELNYLAN 228
                      ******* ****************************************** 
```

Figure 7. Amino acid alignment of the PAL protein in *A. serpens* and *P. patens*.

ClustalW was used to align the newly sequenced *A. serpens* PAL gene and the *P. patens* sequence that was found on NCBI. 90% identity of known *P. patens* PAL gene and proposed PAL gene from sequencing in *A. serpens*.

Re-designed Physcomitrella patens primers

Primers were designed for PR-1, PR-2, PR-3, PR-5, PR-6 and CHS. Investigation into whether these primers will amplify the target genes is still in progress. The gene expression analysis for the CHS gene is shown in figure 10.

Discussion

The two defense genes, CHS and PAL, were successful in amplifying sequences in *A. serpens*. Some of the other primer sets used were able to amplify sequences, but showed many bands in the gel indicating that there was unspecific amplification. It is likely that the other primer sets used were not amplifying the correct target sequence.

The sequence information from the CHS and PAL PW primers was compared to known sequences in *Physcomitrella*. The CHS gene identified in *A. serpens* was 97% identical to the *P. patens* CHS (Fig. 7). The PAL gene identified in *A. serpens* was 90% identical to the *P. patens* PAL gene (Fig. 8). The high percentage of identity confirms that the correct target gene was identified.

Primer design works differently when using RNA vs DNA. In DNA, the target sequence will have a promoter, introns and exons, but mRNA contains only the exons from the original sequence. The *Physcomitrella* genome has been sequenced, but the intron exon slice sites have not been identified. When designing primers for use in RNA, there is a possibility that the primers will not work due to missing introns and promoter.

The PW primers were screened using DNA first, so the issue of not knowing slice-sites is not an issue here. It could be that PR genes not highly conserved enough to amplify in another moss species. The CHS and PAL genes seem to be highly conserved since these primers were able to amplify sequences in *A. serpens*.

The degenerate primers largely did not amplify or the products had many bands suggesting that it was non-specifically amplifying DNA. These primers were designed to be fairly nonspecific but probably didn't work because of too many bases in the primers were non-specific.

The redesigned *Physcomitrella* primers will need to be screened using qRT-PCR to determine if genes are expressed and induced by redesigned *P. patens* primers problem in using RNA and intron/exon splice sites are unknown.

Literature Cited

Loon, L.C. van, M. Rep, C.M.J Pieterse. "Significance of Inducible Defense-Related Proteins in Infected Plants." *Annu. Rev. Phytopathol*, March (2006): 44: 135-62.

Ponce de León, Inés, Juan Pablo Oliver, Alexandra Castro, Carina Gaggero, and Marcel Bentancor. "Erwinia carotovora elicitors and Botrytis cinerea activate defense responses in *Physcomitrella patens*." *BMC Plant Biology* 7.52, 8 Oct. (2007).

Oliver, Juan Pablo, Alexandra Castro, Carina Gaggero, Tomas Cascón, and Eric A. Schmelz. "Pythium infection activates conserved plant defense responses in mosses." *Planta* 230, 24 June (2009): 569-79..

Winter, Peter S., Collin E. Bowman, Philip J. Villani, Thomas E. Dolan, and Nathanael R.

Hauck. (2011) "Systemic acquired resistance in moss: Further evidence for conserved defense mechanisms in plants" Unpublished Manuscript.

Chapter 3: Gene Expression in *Amblystegium serpens*

Background/Introduction

SAR is characterized by an enhanced ability to react to a pathogen attack the next time the plant is attacked. This enhanced ability is called "priming" and it occurs not only in the region that was initially attacked but in tissues in other regions of the plant as well (Durant and Dong 2004). The SAR is a broad resistance response to infection of many pathogens including viruses, bacteria, oomycetes, and fungi. Elicitors can be used to cause the same defense response as pathogens, but do not attack the plant. A commonly used fungal elicitor is chitosan. Chitosan is structurally similar to chitin which is found in structural components of fungal cell walls,. Once the plant is initially attacked, there is a signal sent from the place of infection to other tissues. The exact identity of the signal is unknown, but it is thought to be a lipid signal. Other studies have supported the idea that ethylene, a simple gas, or hydrogen peroxide, ROS as a byproduct of metabolism, as possible components of SAR signaling (Bate et al. 1998). Once SAR is activated, salicylic acid (SA) is needed to establish SAR in distal tissues and activate defense genes there (Ryals et al. 1996). The cells are then primed for another attack in the future, the exact mechanism of how cells do this is not entirely understood. It is believed that priming may involve accumulation of cellular components important in signal transduction and amplification (Conrath 2006). Another study suggests that priming may also include histone acetylation of defense genes (Ng et al.

2005). These activities would not activate the defense genes, but would make the process of responding to an attack much quicker.

An important characteristic of SAR is that not only the tissues around the initial site of infection are protected from another attack, but that tissues in other parts of the plant are protected as well. Previous studies looking at the defense system of *P. patens* have determined that nonvascular plants most likely have SAR for the following reasons, mosses are able to have better defense against pathogens after the first introduction, SA seems to induce heightened defense against infection and PR1 was identified in *P. patens* after infection (Andersson et al. 2005). However, the heightened ability to fight infection in distal tissues was not studied until a recent study at Butler University. A possible cause for the lack of evidence of a heightened response in distal tissues is the growth habit of *P. patens*. It grows in short bushy clumps that grew from one protonema (fig 3.). It would be very difficult to infect one end and determine the response on the other end of the gametophyte. This is why Winter et al. (submitted) used another moss species, *Amblystegium serpens*. *A. serpens* has a creeping growth habit that makes it much easier to demonstrate that the distal end of the gametophyte also has a heightened immunity. Winter et al. (submitted) used *Pythium irregulare* which is a common oomycete that acts as a necrotizing pathogen for many plant species. First they showed that *P. irregulare* was able to infect *A. serpens*, then that the next time the pathogen was introduced the plant was able to defend against the pathogen much more effectively. They also showed that the distal end had obtained this

same resistance to the pathogen. The presence of priming of distal sites to pathogens most likely also occurs in *P. patens* since these are closely related species.

The defense system of mosses seems to be homologous to that of the vascular plants. If mosses and vascular plants both share the SAR response, it is also likely that mosses use the same defense genes as well. PR-1 has already been identified in *P. patens*, but other PR genes may also be present. In this study we designed primers for many different defense genes. The expression levels of CHS and PAL after inoculation with chitosan were studied as well as samples of distal and site of infection tissue.

Methods

Time Course

The moss was subcultured in MS media and 3ul of 1mM chitosan was applied to one end of the moss. At chosen time points (0 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, and 24 hours) the moss was cut in half and frozen in liquid nitrogen. The site of infection and distal ends were collected separately.

RNA extraction

The tissue was ground in liquid nitrogen to disrupt cells. The RNeasy Plant Mini Kit (Qiagen) was used to isolate RNA from the samples. When working with RNA all work

areas were kept RNase-free. The quality of the RNA was determined by electrophoresis described by the Rneasy kit.

Two Step RT-PCR

To analyze gene expression of extracted RNA, RT-PCR was used. The Qiagen RT-PCR kit with Oligo-dt primers was used to convert the RNA to cDNA. The cDNA was used as a template in PCR to amplify the CHS, PAL, and actin genes. Actin was used as a loading control. The PCR products were then run out on an agarose gel using electrophoresis.

Results

CHS timecourse

CHS is expressed in tissues at site of application after initial inoculation as well as the distal site. CHS shows an increase in expression over time and eventually to be down regulated after 12 hours (Fig 7.).

PAL timecourse

Constitutive expression of PAL over all time points (Fig 8.). No difference observed in expression levels between site of application and distal site.

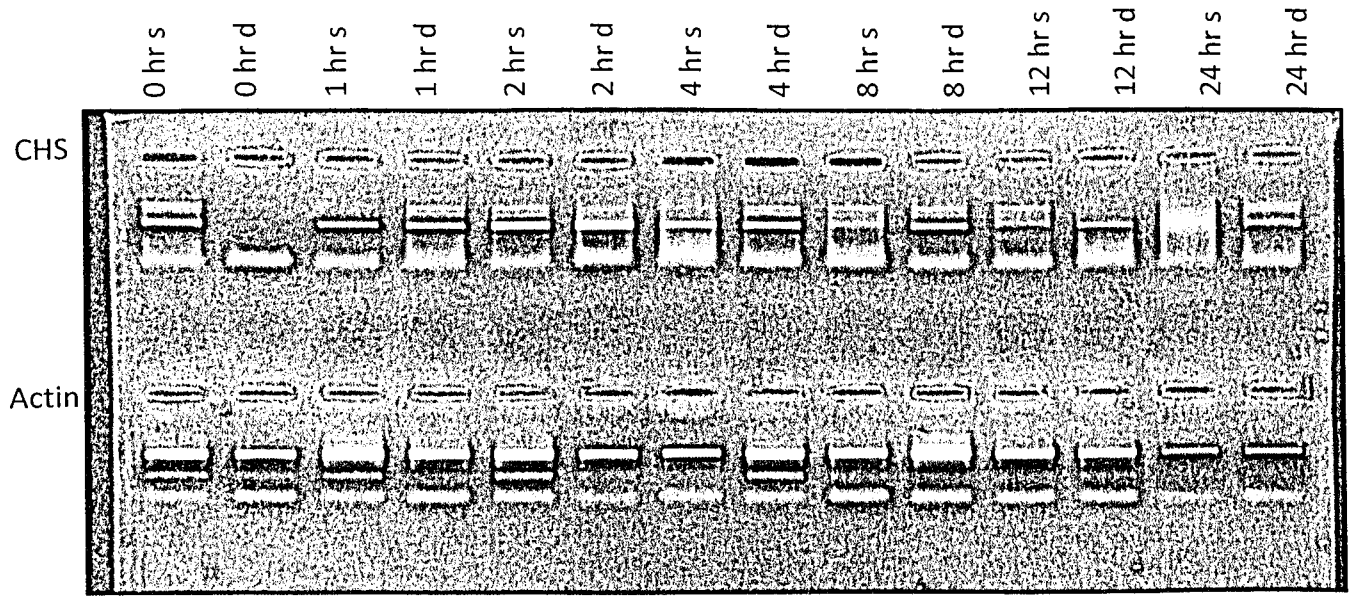

Figure 7. Expression of CHS from 0 hour to 24 hours after chitosan application.

Expression was determined by RT-PCR . The top lane is CHS and the bottom lane is actin.

In the legend, 's' refers to site of application and 'd' to distal site.

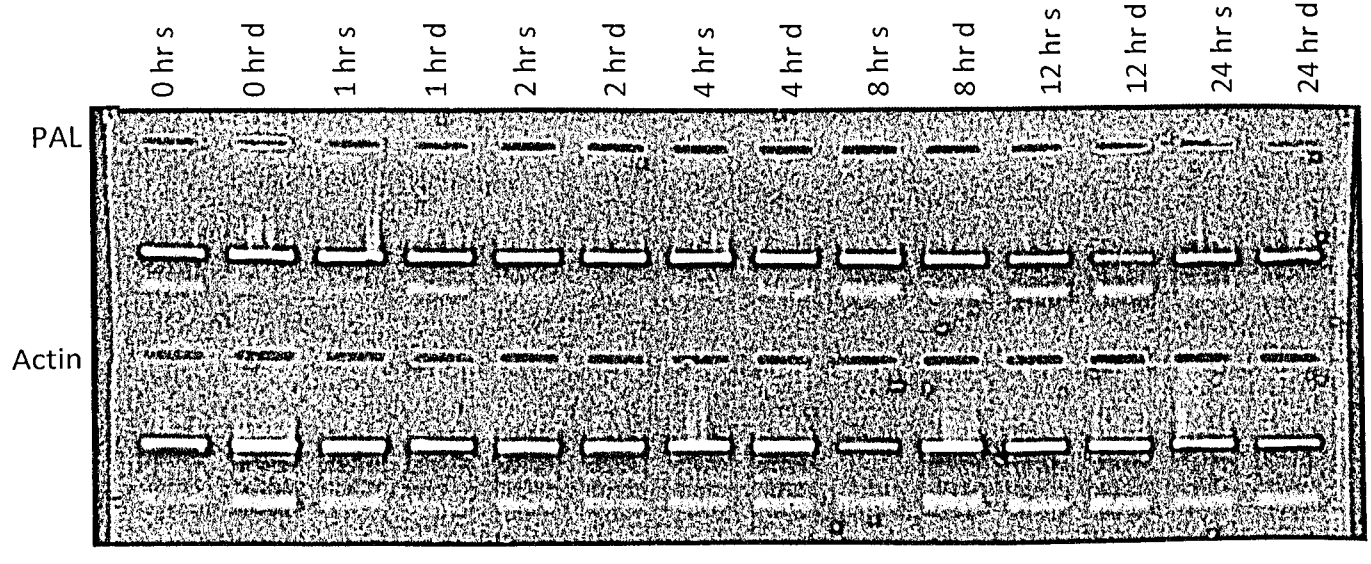

Figure 8. Expression of PAL from 0 hours to 24 hours after chitosan application.

Expression was determined by RT-PCR. The top lane is PAL and the bottom lane is actin.

In the legend, 's' refers to site of application and 'd' to distal site.

Discussion

Defense related genes are expected to be silent or expressed at a low level until turned on by the introduction of a pathogen. For the chitosan time course experiment we would expect for neither CHS nor PAL to be initially expressed but then rapidly turned on.

Previous experiments suggest that chalcone synthase (CHS) is a high conserved defense gene in moss (Fig 6). CHS is expressed within 1 hour of treatment with chitosan. The highest expression is at the 2 hour time point and there is complete disappearance by 8 hours for the site of application and by 12 hours for the distal site. The results for CHS seem to be what is expected for expression of defense genes, although the experiments need to be repeated to fully confirm this pattern in *A. serpens*. It was odd that a band was present at the 0 hour time point. It is possible that the sample was contaminated.

The expected results for gene expression between distal and site of application are that the site of application will show a higher level of expression before the distal site. Since nonvascular plants do not have a vascular system, signals are probably sent by diffusion or capillary action across the outer surface of the plant. Diffusion is slow, and so expression levels in distal tissues may be behind that of the site of application tissues. It is also important that there was also a defense response in distal tissues, because this also supports the idea that SAR is present in nonvascular plants.

The results that we have for the PAL gene is unexpected because the expression was anticipated to be like the defense response pattern described before. One possible reason that PAL appears constitutively expressed is that a study has shown that there is tissue specific expression of PAL. PAL seems to be constitutively expressed in developing xylem tissue but is expressed upon introduction to a pathogen in other tissues (Bevan et al. 1989). Nonvascular plants do not have a vascular system and so do not have xylem tissue. However, some mosses do have rudimentary transport tissues in the central stem that could be related to the conducting tissues in vascular plants (Crandall-Stotler and Bartholomew-Began 2007)

There are three genes that encode PAL. PAL2 is induced by a fungal elicitor and mechanical wounding. PAL3 transcripts only accumulated after mechanical wounding (Bevan et al. 1989). It is possible that the sequence amplified and sequenced in *A. serpens* was the PAL3 gene and so would not respond to a fungal elicitor.

This study needs repeat experiments to determine if the patterns observed are accurate. In order to determine whether PAL is present in *A. serpens*, tissue other than from the central stem should be used due to possible tissue specific for PAL, also the primers for PAL should be redesigned with special care to choose the PAL2 gene. The time course for CHS should be refined to include more time points to determine more exact times.

Literature Cited

Andersson, R. Akita, M. Pirhonen, M. Gammelgard, E. Valkonen, J. 2005. Moss-*Erwinia* pathosystem reveals possible similarities in pathogenesis and pathogen defense in vascular and nonvascular plants. *J. Gen. Plant Path.*71: 23-28.

Bate, N. Rothstien, S. 1998. C_6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. The Plant Journal 16:561-569.

Bevan, M. Shufflebottom, D. Edwards, K. Jefferson, R. Schuch, W. 1989. Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. *EMBO* 8(7): 1899-1906.

Conrath, U. 2006. Systemic acquired resistance. *Plant Signaling & Behavior* 4: 179-184. Review.

Crandall-Stotler, B. J., S. E. Bartholomew-Began. 2007. Morphology of mosses (Phylum Bryophyta). Pages 3–13. In: Flora North America Editorial Committee (eds.), Flora North America, Vol. 27, Bryophytes: Mosses, part 1. Oxford University Press, New York.

Durrant, W. and Dong, X. 2004. Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42:185-209. Review.

Ng DWK, Chandrasekharan MB, Hall TC. 2005. Ordered histone modifications are associated with transcriptional poising and activation of the phaseolin promoter. *Plant Cell* 18:119-32.

Ryals, J. Neuenschwander, U. Willits, M. Molina, A. Steiner, H. Hunt, M. 1996. Systemic acquired resistance. *The Plant Cell* 8:1809-1819. Review.

Winter, Peter S., Collin E. Bowman, Philip J. Villani, Thomas E. Dolan, and Nathanael R. Hauck. (2011) "Systemic acquired resistance in moss: Further evidence for conserved defense mechanisms in plants" Unpublished Manuscript.

Chapter 4: Gene Expression in *Physcomitrella patens*

Background/Introduction

Physcomitrella patens is the model system used for studies of nonvascular plants. It adopts a clumped growth pattern (Fig. 3). The genome has been sequenced fairly recently and some genes have been annotated. An advantage of using *P. patens* for this study, is that several other have been done on defense genes (PR1, CHS, PAL ,LOX). PR-1 has been shown to be expressed in the moss *Physcomitrella* (Ponce de leon et al. 2007). The function of PR-1 has not been fully described. It has been proposed that PR-1 may encode for proteins related to redox reactions with reactive oxygen intermediates. These intermediates may act as local secondary messengers after SA (Maleck 2000). Three defense genes, CHS, LOX and PAL, were also identified in *P. patens* (Oliver et al. 2009). As mentioned earlier, CHS is a defense related gene that is involved in the production of flavonoids, which are known to have anti-microbial and anti-fungal properties. PAL mediates the biosynthesis of phenylpropanoids and salicylic acid (SA). SA has been shown to play a part in signaling in SAR. LOX is a key enzyme in the synthesis of defense related compounds including jasmonic acid (JA) and SA. LOX is also thought to be responsible for activation of PAL. It is likely that other defense genes are present in *P. patens* other than PR-1, CHS, PAL and LOX.

The expected gene expression pattern in *P. patens* for defense genes is in three stages. It starts with no expression or a low level of expression before introduction to a pathogen, an upregulation is seen when the plant is introduced to a pathogen and then

there is a downregulation back to normal levels after a period of time. Gene expression is determined by RT-PCR or qRT-PCR. RT-PCR procedures are used to convert mRNA to cDNA. The cDNA can be used in PCR reactions to determine the presence of target genes with specific primers for those genes. Once the PCR products are run out on an electrophoresis gel, the intensity of the bands indicates how much the gene was expressed. Gene expression results can be compared to determine how the gene is up and down regulated at different times after an inoculation. Quantitative RT-PCR is a new method that has basically completely replaced the original RT-PCR. It works similarly to RT-PCR in that RNA is required and then RNA is reverse transcribed to cDNA. SYBR green is added to the reaction and will only fluoresce when bound to double stranded DNA and so the level of amplification can be measured by fluorescence. qRT-PCR measures the level of amplification at the elongation step of every PCR cycle. The Cq value is the level in which the fluorescence can be measured and this value is important when determining the level of up or down regulation.

This study used qRT-PCR to determine expression of PR-4 and CHS in P. patens following elicitor application. With this knowledge, we will have a better understanding of the timing of the defense response in non-vascular plants.

Methods

Time course

The moss was subcultured in MS media and 30ul of 1mM chitosan was applied to the clump of moss. At time points between 0 and 24 hours, the moss was collected and frozen in liquid nitrogen.

RNA extraction

The tissue was ground in liquid nitrogen to disrupt cells. The Qiagen RNeasy Plant Mini Kit was used to isolate RNA from the samples. When working with RNA all work areas were kept RNase-free. The quality of the RNA was determined by electrophoresis described by the Rneasy kit.

One step qRT-PCR

Quantitative RT-PCR was used in this study. The QuantiFast SYBR® Green RT-PCR Kit was used and the instructions for step up of reactions were followed. Mastermix was made for each RNA sample and three replicates of each qRT-PCR reaction were run. Two primers have been examined, PR-4 and CHS.

Results

PR-4 expression

The general expression pattern expected for defense genes starts out with little or no expression of target gene, after elicitor is introduced the expression is upregulated, then after a period of time the expression is downregulated back to initial levels. A somewhat similar pattern appeared for PR-4 expression (Fig 9.). Expression was normalized using actin. Initially there is a low level of expression, then at the 4.5 hour time point

expression is 2.3 times higher than the expression level of actin. There seemed to be no expression at the 2 hour time point. The expression after 24 hours is downregulated.

CHS expression

The gene expression pattern of CHS shows an upregulation in response to chitosan. There does not seem to be a significant change in expression until the 4.5 hour time point. Expression at the 4.5 hour time point is nearly 2 times the expression of actin. It looks as if there is a downregulation by 24 hours, but since this experiment has not been repeated it may not be a significant downregulation.

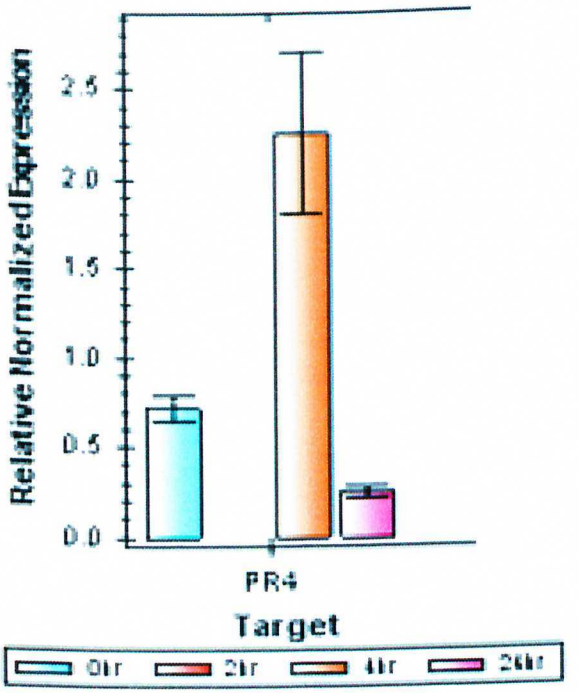

Figure 9. Gene expression analysis of PR-4 in *P. patens* from quantitative RT-PCR. There seems to be a low level of expression before chitosan is introduced but increases expression after chitosan introduction. Expression reaches a high point by 4 hours and drops off between 4 and 24 hours. Unexpectedly, no expression is seen at the 2 hour time point. Expression is normalized using actin as a control.

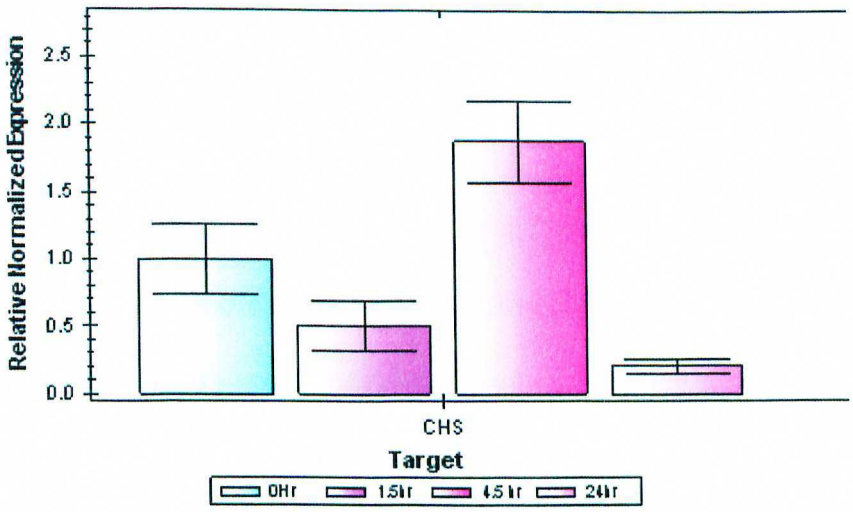

Figure 10. Gene expression analysis of CHS in *P. patens* from quantitative RT-PCR.

There seems to be a low level of expression constitutively, but expression is increased after chitosan is introduced. The highest level of expression is at the 4.5 hour time point and decreases sometime after this time point. Expression is normalized using actin as a control.

Discussion

PR-4 genes encode for chitinases. They are enzymes that cleave poly-β-1,4-Nacetylglucosamine (chitin) (Loon et al. 2006). PR-3 and PR-4 genes would target fungi, oomycetes, and possibly herbivorous insects and nematodes since these organisms use chitin as a structural component (Loon et al. 2006). Chitosan, which is similar in structure to chitin, should induce PR-4. There seems to be a significant difference between expression levels at the 4.5 hour time point and 0 hour time point suggesting that chitosan does induce PR-4 expression. This experiment does need to be repeated to confirm that there is an upregulation in PR-4 expression after introduction to an elicitor. When this experiment is repeated, it is important to note the 2 hour time point because no expression is unexpected.

CHS is a defense related gene that is the first enzyme in the pathway to producing flavonoids, which are known to have anti-microbial and anti-fungal properties (Ponce de Léon et al. 2007). In this experiment it took around 4.5 hours for highest level of expression. As seen in chapter 3, in *A. serpens* it took 1 hour for induction of CHS and the highest level of expression was at the 2 hour time point. These differences could be attributed to using two different methods, RT-PCR and qRT-PCR, of determining gene expression. It could also be fundamental differences in how the two species of moss respond to chitosan.

The primers for the other PR genes have been redesigned (described in chapter 2). These primers, PR-1, PR-2, PR-3, PR-5 and PR-6, will be screened using chitosan treated *P. patens*.

Literature Cited

Loon, L.C. van, M. Rep, C.M.J Pieterse. "Significance of Inducible Defense-Related Proteins in Infected Plants." *Annu. Rev. Phytopathol*, March (2006): 44: 135-62. Review.

Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, et al. 2000. The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* 26:403–10

Oliver, Juan Pablo, Alexandra Castro, Carina Gaggero, Tomas Cascón, and Eric A. Schmelz. "Pythium infection activates conserved plant defense responses in mosses." *Planta* 230, 24 June (2009): 569-79..

Ponce de León, Inés, Juan Pablo Oliver, Alexandra Castro, Carina Gaggero, and Marcel Bentancor. "Erwinia carotovora elicitors and Botrytis cinerea activate defense responses in *Physcomitrella patens*." *BMC Plant Biology* 7.52, 8 Oct. (2007).

Final Conclusion

An impressive amount of information regarding plant-pathogen interactions in vascular plants has been collected over the past few decades. This has led to our understanding of genes involved in defense, the role of hormones, and the mechanism of HR and SAR. However, very little is known about these interactions in non-vascular plants. The goal of the current research was to identify defense genes in moss and characterize their expression following pathogen inoculation.

My hypothesis was that homologous defense genes would also be present in nonvascular plants. This study supports this hypothesis through several experiments. First, I was able to successfully amplify and sequence two defense-related, CHS and PAL, in *A. serpens*; these sequences had high percent identity with similar genes in *P. patens*.

To be functional in defense, defense-related genes are expected to be silent or expressed at a low level until turned on by the introduction of a pathogen. For the chitosan time course experiment we would expect for neither CHS nor PAL to be initially expressed but then rapidly turned on. The results for CHS followed the expected pattern, although the experiments need to be repeated to fully confirm this pattern in *A. serpens*. The results of the PAL time course experiment were unexpected, since PAL was constitutively expressed. Two possible reasons for these unexpected reasons are that a study has shown that there is constitutive expression of PAL in some tissues and inducible expression in other tissues in vascular plants or the wrong PAL was amplified

by the primers. Perhaps a different PAL gene in the moss genome is induced by pathogen exposure.

A key characteristic of SAR, is that the distal tissues will also show "priming" against subsequent pathogen attack. It is expected that the site of application will show a higher level of expression before the distal site. Since nonvascular plants do not have a vascular system, signals are probably sent by diffusion or capillary action on the outer surface of the plant. If signals are sent by diffusion the expression levels in distal tissues may lag behind that of the site of application tissues due to diffusion being slow. This pattern was observed (Fig 7.) and supports the presence of SAR in nonvascular plants. These experiments do need to be repeated to confirm the pattern.

Gene expression of PR-4 and CHS has been studied so far in *P. patens*. There seems to be a significant difference between the maximum level of expression and 0 hour time point suggesting that chitosan does induce PR-4 expression. This experiment does need to be repeated to confirm that there is an upregulation in PR-4 expression after introduction of an elicitor. For the CHS experiment, it took around 4.5 hours for highest level of expression. As seen in chapter 3, in *A. serpens* it took 1 hour for induction of CHS and the highest level of expression was at the 2 hour time point. These differences could be attributed to using two different methods, RT-PCR and qRT-PCR, of determining gene expression. It could also be fundamental differences in how the two species of moss respond to chitosan.

Future directions

More experiments need to be performed to provide enough evidence for the rest of the hypothesis that PR genes are present in nonvascular plants. The primers for some PR genes have been redesigned (described in chapter 2). These primers, PR-1, PR-2, PR-3, PR-5 and PR-6, will be screened using chitosan treated *P. patens*. PR-4 and additional PR genes that seem to be expressed in *P. patens*, should be sequenced to confirm the correct targets are amplified. If some PR genes cannot be amplified using primers, it may be necessary to use a cDNA library to pull these genes out of the transcriptome.

The experiments that have already been done need to be repeated so that statistical studies can be performed and to determine if the gene expression patterns observed are accurate. It may also be beneficial to repeat the *A. serpens* experiments using qRT-PCR so that data between *A. serpens* and *P. patens* can be compared.